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14. ABSTRACT

The Mustards Consortium has utilized both in vivo and in vitro models simultaneously to continue to elucidate mustard gas pathophysiology. In previous work done by the MC it was found that CEES, the mustard analogue, induced oxidative stress and was its primary mechanism of action. Consequently, NAC (N-acetyl cystiene) was found to be protective as a prophylaxis and treatment. A combination of a water and fat soluble antioxidant encapsulated in a liposome (STIMAL) was found to have the best ameliorative effect against CEES. We have initiated development of next generation STIMAL, in order to optimize its ameliorative effect. The mechanism of action of the antioxidants is suspected to be primarily by their effect on redox regulated pathways. In an effort to elucidate the mechanism of action of the antioxidants and the pathophysiology of mustards, profiles are being developed for: gene expression and antioxidant levels, as well as biochemical pathways. The first known histological comparison between CEES and sulfur mustard was carried out. Two new rat lung models were developed for the administration of sulfur mustards in preparation for efficacy testing of STIMAL against sulfur mustards. Pulmonary fibrosis was demonstrated in both guinea pig and rat lung models.

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SECTION 1: Milton G. Smith, M.D. – Director Amaox, Ltd.

An Overview of the Continuation of the Work of the Mustard Consortium for the Use of the Free and Liposome Encapsulated Antioxidants as a Counter Measure to Mustards

Introduction and Body

An overview of the Mustard Consortium work 2003-2006

Two major problems are faced by the threat of the use of mustard gas on the battlefield: 1) the absence of an antidote; and 2) the inability to detect sulfur mustard at subclinical levels.

One of the primary organs affected by exposure are the lungs. Known complications are: ARDS, pulmonary fibrosis, stenosis of the bronchial tree. The absence of an ameliorative agent for mustards is compounded by the inability to diagnose exposure in the person who is asymptomatic or showing minimal symptoms. Subclinical exposures may have long term complications (e.g. Gulf War syndrome (?)). Tests have been developed for detection of SM specifically, but none exists for testing for multitude of agents rapidly. In this new body of work we are providing the foundation for a better understanding of the pathophysiology sulfur mustard and its analogue CEES (2-chloroethyl ethyl sulfur); as well as potential treatment and diagnostic capability.

Antioxidants have been found to be an ameliorative agent for CEES, the mustard analogue. Liposome encapsulation of the antioxidants (STIMAL) significantly enhances the ameliorative effect.

In this phase of the research we began preparation for efficacy testing of STIMAL in the animal models with sulfur mustards. Dr. Sciuto has developed an aerosol inhalation model, which simulates the inhalation of SM under physiological conditions (similar to what would occur on the battle field).

The LCt10 at 1250 ug of SM was determined. An LCt50 has not been achieved as yet. Protein levels increased 4 fold at 0.5 to 24 h at the 1250 ug dose, suggesting a breach of the air/blood barrier.

Mr. Anderson has used the experimental design that was developed by Dr. Ward, which is the instillation of the agent into the deep lung. In the Anderson studies it was determined the SM was equivalent to the CEES dosage (approximately 1:6 ratio) that was used by Dr. Ward in the rat lung model. This information will increase the relevancy of the work done by civilian investigators that are restricted to the use of CEES. Rat spleen, kidney, and liver that had been exposed to SM (in vivo) were sent to Doctors Stone and Crawford.

Dr. Stone's lab served as a core facility for the consortium that performed analysis of oxidative stress reactions in tissues. Glutathione levels were assayed in tissues sent from Mr. Anderson's laboratory. In the CEES exposed organs GSH was decreased. In contrast, the SM exposure resulted in a decrease in the splenic GSH, but essentially no change in the liver and kidney. The

decreased GSH levels would imply that oxidative stress is occurring in these tissues. There was essentially no change in the tocopherol levels. These results imply that CEES produces systemic oxidative stress at a greater level than does SM.

Dr. Stone has been in the process of optimizing a several formulations of STIMAL. Out of the several formulations that are developed, a few will be tested in the efficacy studies that will be performed by Dr. Sciuto and Mr. Anderson. Dr. Stone has determined that polymyxin B (an antibiotic) can inhibit the exacerbating effects LPS in macrophages exposed to CEES. This finding may be significant in the treatment of skin that is exposed to SM. It is unknown if polymyxin B will have the same inhibitory effect on SM that it has shown for CEES.

Dr. Ward determined in the rat lung model, that the bronchoalveolar lavage fluid (BAL) contained inflammatory mediators that increased after CEES administration. IL- 2 β peaked at 2 hours; whereas TNF- α, MP-2, CINC-1 peaked at 4 hours. Liver enzyme release, an indicator of injury, peaked at 4-6 hours; there was essentially no change in kidney function. Selected genes associated with apoptosis (BAX, Egr1, Hspb1, HSP90-Rik, Nos2, Ccl2) were increased at 4 hours. Pulmonary fibrosis, a known complication of SM, was examined in this phase of the study. It was found that lung collagen levels are increased within 3-4 hours of CEES exposure. The question of whether CEES can compromise the innate immunity of the lung has also begun to be examined.

Dr. Das has continued to elucidate a biochemical pathway in the guinea pig lung model that may be one of the contributing factors to the complication of ARDS. Cholinephosphotransferase (CPT) gene expression and enzyme activity were decreased. There is an inverse correlation between ceramide production and CPT gene expression. The same inverse correlation exists for ceramide and the CPT enzyme activity. Selected gene expression was examined. Increases were noted for IL-α, EOTAXIN, MP1 γ, IFN-γ, TNF-α, NFkB (Light), PDGF-BB, FGF7, IGFBP-I. Pulmonary fibrosis occurred at 7 days.

Dr. Crawford has developed one of the two core facilities (Dr. Stone being the other). His core was responsible for the genomic and proteomic analysis of samples sent from the other members of the group. The data from this analysis will be made available to the group members via a secure web site that has been developed. A toxic gene micro array for rat and mouse has been developed that will assist in the identification of gene expression for potentially several chemical weapons. A protocol is being developed for the isolation of mRNA from blood, spleen, and lung. It is anticipated that obtaining this base line data will result in a new robust diagnostic tool that could diagnose several chemical WMD.

Key Research Accomplishments

- Animal models to test the efficacy of STIMAL against sulfur mustard have been developed; two different methods of delivery of STIMAL will be utilized along with two different methods of sulfur mustard delivery.
- Development of the next generation of STIMAL.
- Sulfur mustard and CEES equivalent doses have been determined (about a 1:6 ratio); histological comparisons were done. Glutathione and tocopherol levels were assayed in kidney, lung and liver.

- Observation of pulmonary fibrosis in rat and guinea pig lung models.
- Observation of BAL inflammatory mediators.
- Gene expression in guinea pig and rat lung.
- Development of tox gene micro array and web access for group data.

Reportable Outcomes

- See the individual investigator reports.
- A Bioscience presentation was made on the role of oxidative stress in CEES pathophysiology.
- A Mustard Consortium meeting was held during the Bioscience meeting- 2004.
- A Mustard gas consortium meeting was held in Orlando FL 2005 (October 6-9)
- A presentation was made at Bioscience 2006 Medical defense meeting held at Hunt Valley MD (June 4-9).
- Presentations were given by all of the members of the group that facilitated an understanding of the individual projects and how they fit it to the overall research effort.
- McClintock, S. D., L. M. Hoesel, et al. (2005). Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol.
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- Smith, M., Stone, W., Crawford, K., Ward, P., Till, G., Das, S.,: Features, Antioxidant Liposomes- A New Treatment for Mustard Gas With the Potential to Substantially Reduce the Threat Posed by Chemical, Biological and Radiological Agents. Janes.com, Feb 2003

Conclusions

The biological effects at the cell, organ and systemic levels, are being described for sulfur mustard (SM) as well as the protective effects of liposome encapsulated antioxidants (STIMAL). STIMAL is able to protect against the deleterious effects of SM on the cellular and organ redox balances, thereby attenuating the injurious and destructive biological impact of SM. Current studies also suggest that STIMAL can be protective even after lung exposure to SM.

References

See individual reports

Appendices

None

SECTION 2: Peter A. Ward, M.D., University of Michigan Medical School

An Overview of the Continuation of the Work of the Mustard Gass Consortium for the Use of the Free and Liposome Encapsulated Antioxidants as a Counter Measure to Mustard.

Abstract

Acute lung injury in rats following airway delivery of CEES is associated with loss of distal lung barrier function (resulting in alveolar hemorrhage) and an intense inflammatory response, which is lung-damaging. These acute lung injury parameters are attenuated by neutrophil depletion or complement blockade. Injuring the redox balance in lung after exposure to CEES by administration into lung of liposomes containing antioxidant compounds is highly protective even when delivery of liposomes is delayed by at least 1 hr. CEES-induced lung injury is progressive, as manifested by development of interstitial fibrosis which seems to peak at three weeks. Whether STIMAL will attenuate development of lung fibrosis is currently unknown.

Introduction

As is well known, mustard gas [bis (2-chloroethyl ethyl) sulfide], also known as sulfur mustard (HD), has long been known to be a vesicant in humans and, when inhaled, causes extremely lung damaging reactions (1-3). In human survivors, progressive lung dysfunction due to pulmonary fibrosis is well documented (4). Not unexpectedly, HD is radiomimetic, teratogenic and mutagenic (5,6). Currently, there is no effective therapy for either the vesicant-inducing properties of HD or for the outcomes that can lead to acute and progressive lung injury and death.

2-chloroethyl ethyl sulfide (CEES) is less toxic than HD and can be used in the absence of facilities required for HD studies. In rats CEES has been shown to induce acute lung injury in a dose-dependent and time-dependent manner (7). CEES-induced acute lung injury is complement and neutrophil-dependent, suggesting that some of the CEES-induced injury is due to engagement of the inflammatory response in lung in an unknown manner (7). Furthermore, lung injury is attenuated after intravenous treatment with the anti-oxidant, N-acetylcysteine (NAC), or airway delivery of anti-oxidants or anti-oxidant enzymes (7). These data have suggested that CEES compromises the redox potential in lung, putting it at risk of oxidant-mediated injury.

Liposomal delivery of drugs or chemical compounds is a way to achieve high intracellular levels of a desired compound in tissue macrophages (8-10). In lung, airway delivery of liposomes results in macrophage uptake of liposomes by a phagocytic pathway (11-13). As far as is known, liposomes are not internalized by any other lung cells. Our recent studies suggest that intrapulmonary delivery of liposomes containing anti-oxidants are strongly protective of CEES-induced acute lung injury, even though little is currently known about how CEES produces acute and progressive lung injury.

Body

Experiments performed over the past years (Aug 03 – Aug 06) have provided evidence that depletion of the complement system as well as the intratracheal instillation of liposomes containing anti-oxidants or reducing agents, or liposomes containing the combination of both, results in greatly reduced lung injury. Histological analyses following airway instillation of CEES have revealed intra-alveolar hemorrhage, edema and intra-alveolar accumulates of

macrophages, neutrophils and mononuclear cells in lung by 6 hour post injury. Increased fibrin and collagen deposition in alveolar walls, as defined by trichrome staining of tissue sections, was seen as early as 24 hours after instillation and by 3 weeks resulted in dense deposits of fibrin and extensive confluent collagen deposits together with collapse of alveolar structures. This resulted in the histologic appearance of "honeycombing" of the lung, indicating lung fibrosis and alveolar collapse. A manuscript entitled "*Attenuation of Half Sulfur Mustard Gas–induced Acute Lung Injury in Rats*" has been published in the *Journal of Applied Toxicology*.

Continuing studies involve four different areas:

1. Patterns of inflammatory mediators after CEES-induced lung injury. Bronchoalveolar lavage fluid (BALF) levels of pro-inflammatory cytokines/chemokines are assessed by ELISA. IL-1β was found to peak 2 hours after lung exposure to CEES, while TNFα, MIP-2 and CINC-1 levels peaked at 4 hours. A time course of lung inflammatory mediators involving superarray analysis is currently underway. RT-PCR validation of selected genes is also in progress.
2. Long-term effects (fibrosis) after CEES injury. Using biochemical assays (hydroxyproline content), lung collagen content is significantly increased 3 and 4 weeks after CEES exposure. This appears to mimic what happens in humans exposed to mustard gas. Possible beneficial effects of liposomes containing reducing agents (tocopherol and NAC) will be assessed in the CEES model, in conjunction with Dr. W. Smith (E. Tennessee State U.) and Dr. S. Das (Mehary Medical School).
3. Pulmonary clearance of *Pseudomonas aeruginosa* in CEES treated rats. It is not known if exposure to CEES compromises the ability of the lung to clear bacteria. In order to simulate a clinical situation (bacterial superinfection in the ICU), *P. aeruginosa* will be administered intratracheally at certain time points after CEES administration to induce acute bacterial pneumonia. Bacterial clearance will be assessed by content of colony forming units (CFU) in lung homogenates and in whole blood. Possible beneficial effects of liposomes containing reducing agents (NAC/GSH) will be determined.
4. Systemic effects of intratracheal application of CEES on organ function. Preliminary data suggest that liver enzymes (AST, ALT) peak in the serum 4 and 6 hours after lung exposure to CEES, with return to normal values within 48 hours, indicating transient liver damage after airway delivery of CEES. In contrast, renal parameters (CREA, BUN) were unchanged. Possible protective effects of airway instillation or intravenous injection of liposomes containing reducing agents (NAC/GSH) are in progress.

Key Research Accomplishments (Aug 03 – Aug 06)

- Ability of STIMAL (antioxidant liposomes) to greatly attenuate acute lung injury after CEES, even when liposomal administration is delayed following exposure to CEES.
- Appearance in BAL fluids of cytokines and chemokines (IL-1b, TNFa, MIP-2, CINC-1) after lung exposure to CEES.
- Evidence that lung exposure to CEES also includes acute liver injury.
- Biochemical evidence that lung exposure to CEES results in progressive pulmonary fibrosis based on histopathology and biochemical evidence.

Reportable Outcomes

1. "Systemic Effects of CEES (Half Sulfur Mustard Gas) after Intratracheal Instillation", L.M. Hoesel, A.D. Nielerbichler, S.D. McClintock, J.V. Sarma, P.A. Ward, presented at the SHOCK Society Meeting (San Marcos Island, Florida, June 05)
2. "Protective Effects of Anti-Oxidant Liposomes on Acute Lung Injury after CEES", presented by P.A. Ward at Bioscience 2004, Hunt Valley, Maryland May 18-20, 2004.
3. "Protective Effects of Antioxidant Liposomes in Lung Injury", poster presented by P.A. Ward and G.O. Till at the Mustard Gas Consortium, Hunt Valley, Maryland, May 19, 2004.
4. "Protective Lung Effects of STIMAL", presented by P.A. Ward to Congressional Staffers at Summit Meeting, October 18, 2004, Capital Building, Washington DC.
5. Discussion of STIMAL Strategy for Protection Against CEES in Induced Acute Lung Injury, by P.A. Ward and other consortium members in Plenary Session at Bioscience 2004, Hunt Valley, Maryland, May 19, 2004.
6. Poster presentation: L.M. Hoesel, A.D. Niederbichler, S.D. McClintock, J.V. Sarma, P.A.Ward. "Systemic effects of CEES (Half Sulfur Mustard Gas) after intratracheal instillation" 28th Annual Conference on Shock, Marco Island, FL, June 4-7, 2005
7. Update on previous and current projects involving CEES-induced lung injury in rats Mustard Gas Consortium Meeting, Orlando, FL, October, 6-9, 2005, Oral presentation.
8. Protective Effects of Anti-Oxidants Liposomes in CEES and other models of Acute Lung Injury Bioscience 2006 Medical Defense Review, Hunt Valley, MD June 4- 9, 2006, Oral Presentation
9. Poster presentation: L.M. Hoesel, M.J. Pianko, H. Yang, W.L. Stone, M.G. Smith, P.A. Ward. "Liposomes containing Anti-oxidants prevent pulmonary fibrosis in Half-Sulfur Mustard Gas induced lung injury" Bioscience 2006 Medical Defense Review, Hunt Valley, MD, June 4- 9, 2006
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Conclusions

Administration of CEES into rat lung produces both acute and progressive lung injury. The former is characterized by an acute inflammatory response associated with a large lung leak (alveolar flooding with plasma components) and accumulation of neutrophils and mononuclear cells. These changes are associated with the appearance of chemokines and cytokines. Airway instillation of STIMAL (liposomes containing antioxidants) together with CEES results in greatly attenuated lung injury, even if STIMAL intervention is delayed for 1 hr after instillation of CEES. CEES also causes progressive lung injury (pulmonary fibrosis), although the causes for this are not known, and it is not known if STIMAL will prevent this complication.

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Appendices

1. McClintock paper of 2002.
2. McClintock paper of 2006.
3. SHOCK Society abstract.

SECTION 3: William L. Stone, Ph.D., East Tennessee State University

Optimization of Antioxidant Liposomes for Treating 2-Chlorethyl Sulfide (CEES) Toxicity

Introduction

We are exploring the hypotheses that: (1) oxidative stress contributes to mustard gas and 2-chloroethyl ethyl sulfide (CEES) toxicity and; (2) antioxidant liposomes are a potential countermeasure. Considerable evidence suggests that mustard gas toxicity is associated with an increased generation of damaging free radical production [1-4]. Antioxidant liposomes may, therefore, provide a unique therapeutic strategy for mustard gas exposure because: (a) the antioxidants are nontoxic and could, therefore, be used at the earliest stages of toxicity (b) the liposomes themselves are composed of nontoxic, biodegradable and reusable phospholipids; (c) liposomes are preferentially taken up by the reticuloendothelial system which is an early target of mustard gas toxicity; (d) chemical antioxidants are relatively inexpensive and a wide range of nontoxic commercial antioxidants are available. The long term goals are to further define the mode of action of mustard gas and to develop a therapy using liposomes containing both lipid and water soluble antioxidants. These goals are consistent with the USAMRMC Medical Chemical Defense Research Program interests in the area of defense against chemical agents.

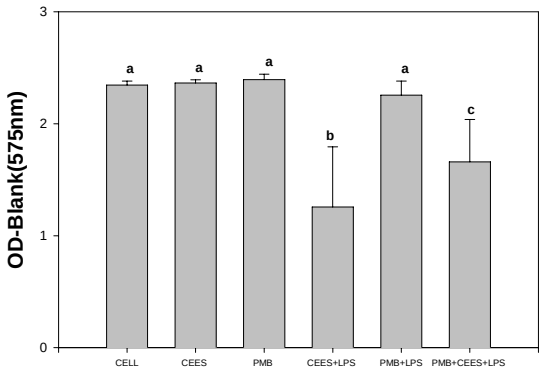
Body

Task: Optimize: (a) the antioxidant composition of liposomes for treating CEES toxicity in cell models and; (b) the stability of antioxidant liposome preparations.

In order to advance the development of antioxidant liposomes for eventual military application it is essential that we scale-up our previous liposome preparation and use a preparation technique in which sterilization can be more easily (and reproducibly) accomplished. We have now developed the ability to prepare and characterize antioxidant liposomes in quantities sufficient to supply other members of the Mustard Consortium.

A Model M-110L Microfluidics instrument has been recently acquired and used for the preparation of unilamellar antioxidant liposomes containing 6.6 mole percent RRR-alpha-tocopherol (as well as 66.6, 26.5 and 0.66 mole percent of soy lecithin, cholesterol and phosphatidyl serine, respectively). The liposomes were characterized by measuring: 1) particle size distribution with a dynamic light scattering Model 380 Nicomp particle analyzer; 2) tocopherol content. Our data indicates that the vitamin E content was stable over a three week storage (at 4°C) period and did not diminish with up to five passes through the

Figure 1. Polymixin B (PMB) Inhibits CEES Toxicity (after 18 hours) to LPS Stimulated RAW 264.7 Macrophages. Cytoxicity was Measured by the MTT Assay. Means labelled with different letters are significantly different (p<0.05).



Microfluidics instrument. The mean liposome diameter decreased with increasing passes (as expected) through the fluidizer. The liposome preparation remained unilamellar over a three week period (at 4°C). In summary, we can now prepare large (100 ml) batches of antioxidant liposomes that have sufficient stability for shipping and further testing. We are now in the process of preparing a liposome preparation for use by both Dr. Peter Ward's group and Dr. Dana Anderson group (USAMRICD) for in vivo testing.

We have also initiated studies using a variety of antioxidant liposomes in treating CEES toxicity in cell models. To date we have found that GSH-liposomes, NAC-liposomes and alpha-tocopherol-liposomes are effective in preventing CEES toxicity to RAW264.7 macrophages [5].

Task: Develop Counter Measures to Treat CEES Toxicity

Polymyxin B Inhibits CEES toxicity in stimulated macrophages: In our previous published work, we reported that very low levels of lipopolysaccharide (LPS or endotoxin) were capable of enhancing the cytotoxic effects of CEES [5, 6]. LPS is ubiquitous and is present in serum, tap water, and dust. Military and civilian personnel would always have some degree of exposure to environmental LPS, which could increase the toxicity of mustard gas. In addition, there is always the possibility of purposeful LPS exposure.

LPS binds to CD14 and initiates a complex signal transduction pathway also involving the Toll receptor family. In addition, LPS activates NFκB, which activates the transcription of NO synthase. In preliminary results (see Figure 1 above), we have found that murine RAW264.7 macrophages treated with LPS (50 ng/ml) and CEES (500 μM) for 18 hours show a cytotoxicity that is partially blocked by 5 μg/ml Polymyxin B (PMB), a cationic antibiotic capable of binding and blocking the action of LPS. Cytotoxicity in figure 1 was measured by the MTT assay in which a higher OD at 575 nm indicates greater degree of cell viability.

Figure 2. Nitric Oxide Production in LPS Stimulated RAW 264.7 Macrophages is Blocked by Polymixin B (PMB). Means with different letters are significantly different ($p < 0.01$).

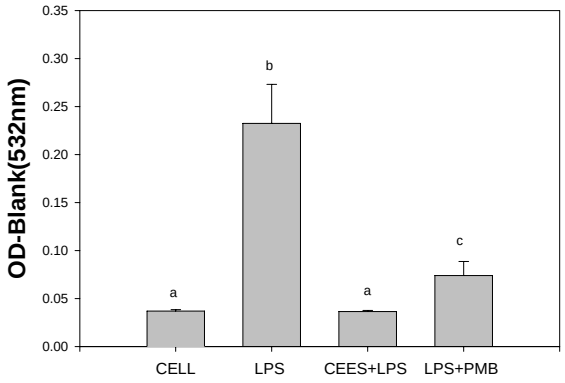
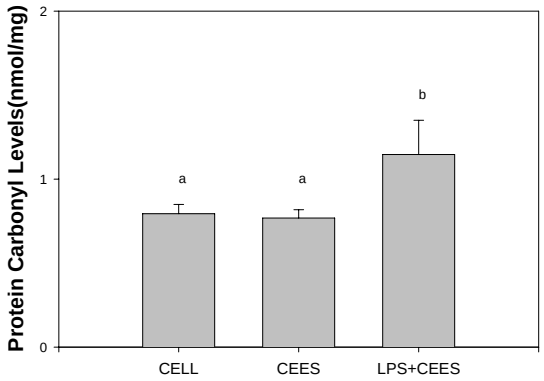


Figure 3. Protein Carbonyl Levels in RAW 264.7 Macrophages Treated with CEES or CEES plus LPS. Means with different letters are significantly different ($p < 0.01$).



Neither CEES nor PMB were cytotoxic alone. As shown in Figure 2, LPS (50 ng/ml) also induced the production of nitric oxide via inducible nitric oxide synthase (iNOS) in RAW264.7 macrophages and this induction could be blocked by polymyxin B pretreatment. These data suggest that polymyxin B could be useful in minimizing the toxicity of mustard gas.

Task: Study the Molecular Mechanisms(s) Underlying CEES Toxicity

Oxidative stress associated with CEES toxicity in Stimulated Macrophages: In further support of the hypothesis that oxidative stress is important in CEES toxicity, RAW264.7 macrophages simultaneously treated with CEES (500 M) plus LPS (10 ng/ml)) for 18 hours were found to have significantly elevated levels of protein carbonyls (see Figure 3). CEES alone was not sufficient to cause an elevation in carbonyl content. Protein carbonyls are an excellent and stable indicator of oxidative stress. Protein carbonyls were measured by an ELISA kit.

Similarly, only the combination of LPS with CEES was sufficient to reduce the levels of GSH in RAW264.7 macrophages (see Figure 4). GSH is the major intracellular antioxidant and loss of this key antioxidant indicates oxidative stress. GSH was measured by a highly sensitive fluorometric technique [7].

Figure 4- The Effect of CEES or CEES plus LPS on GSH Levels.
Means with different letters are significantly different (p<0.01).

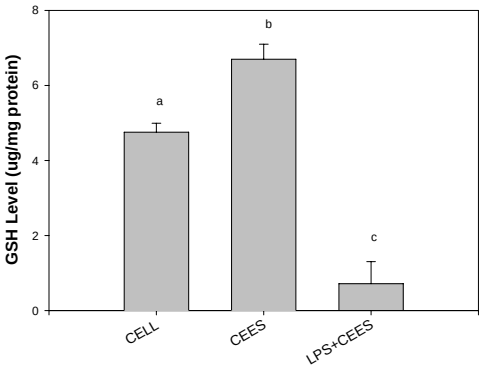


Figure 5- The Effect of CEES and CEES plus LPS on Cell Thiol Levels.
Means with different letters are significantly different (p< 0.05).

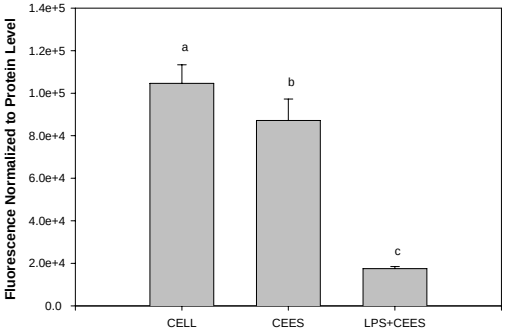


Figure 5 also shows that the total thiol levels in RAW264.7 macrophages were decreased only by treatment with CEES plus LPS.

Task: Determine how the antioxidant status of the animal models is influenced by administration of the antioxidant liposomes (with and without CEES treatment).

Dr. Dana Anderson has sent my laboratory plasma, liver, kidney, spleen and lung samples from rats exposed to CEES or mustard gas. In total, tissues from 58 rats have been analyzed for tocopherols (alpha- and gamma-tocopherols) and GSH levels. These data will be summarized and reported by Dr. Dana Anderson. An example of the data report provided to Dr. Anderson is provide in the Appendix.

We are now preparing to assay the levels of protein carbonyls in these samples and acquired the necessary equipment and supplies for this assay. A graduate student has also initiated a proteomic analysis of some of these samples (2-D gel electrophoresis).

Key Research Accomplishments

- Antioxidant liposomes have been prepared and characterized using large scale techniques and can now be sent to other members of the mustard consortium.
- A number of the antioxidant liposome preparations have been shown to be effective in preventing CEES toxicity to RAW 264.7 macrophages.
- Very low levels of polymyxin B were found to inhibit CEES toxicity in LPS stimulated macrophages suggesting that in could be useful in treating mustard gas toxicity.
- Oxidative stress was found to be associated with CEES toxicity in LPS stimulated 264.7 macrophages as indicated by increased protein carbonyl levels and decreased levels of intracellular GSH and total thiols.

Reportable Outcomes

- Two manuscripts describing the use of antioxidant liposomes and their use in preventing CEES toxicity have been published:
    - Stone, W.L., Smith, M.: Therapeutic Uses of Antioxidant Liposomes, Molecular Biotechnology, 27: 217-230 (2004)
    - Stone, WL, , Qui, M, , Yang, H, and Smith, M: Lipopolysaccharide Enhances the Cytotoxicity of 2-Chloroethyl Ethyl Sulfide, Bioscience 2004 Proceedings, US Army Medical Defense, Chapter 236, pages 1-9. (2004))
- A third publication has been submitted describing the role of oxidative stress in ricin toxicity
    - Suntres, Z, Stone, WL, Smith, MG: Ricin-Induced Toxicity: The Role of Oxidative Stress (submitted).
- A book chapter on Blister agents and oxidative stress is “in preparation”
    - Smith, M, Das, S, Ward, P, Suntres, Z, Crawford, K and Stone, WL: Blister Agents and Oxidative Stress, In: CHEMICAL WARFARE AGENTS: Chemistry, Pharmacology, Toxicology and Therapeutics, CRC Press (in preparation)

Conclusions

Our primary long-term goal is to develop an effective counter measure to mustard gas. Our results to-date reinforces the notion that oxidative stress is an important component of mustard gas and CEES toxicity. In addition, we have found that both water and lipid soluble antioxidants can be effective in reducing CEES toxicity. Collectively, these data suggest that antioxidant liposomes should be an effective counter agent to mustard gas toxicity. Our in vitro data support this hypothesis but further in vivo work will be required. With this in mind, we have developed

the ability to prepare large (100 ml to liter) preparations of antioxidant liposomes and initiated quality control procedures for their pharmacological characterization.

We have also advanced our understanding of the molecular mechanisms underlying CEES toxicity. In particular, we have found that inflammatory agents increase the toxicity of CEES in our in vitro model systems. This knowledge has enabled us to suggest new counteragents. In particular, we have found that polymyxin B was effective in partially blocking the enhanced CEES toxicity observed in LPS-stimulated macrophages.

A comprehensive proteomic approach to understanding the toxicology of mustard gas, CEES and toxic industrial chemicals (TICS) is now technologically feasible. We, along with other members of the Mustard Consortium, have initiated such an approach. My laboratory will place particular emphasis on the newly emerging science of redox proteomics and its applications in developing countermeasures to weapons of mass destruction.

References

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3. CM Arroyo, RL Von Tersch, CA Broomfield: **Activation of alpha-human tumour necrosis factor (TNF-alpha) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulphide (H-MG)**. *Human and Experimental Toxicology* 1995, **14**:547-53.
4. NM Elsayed, ST Omaye, GJ Klain, DW Korte, Jr.: **Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection**. *Toxicology* 1992, **72**:153-65.
5. WL Stone, M Qui, H Yang, M Smith: **Lipopolysaccharide Enhances the Cytotoxicity of 2-Chloroethyl Ethyl Sulfide**. *Bioscience 2004 Proceedings* 2004, **Chapter 236**:1-9.
6. WL Stone, M Qui, M Smith: **Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide**. *BMC Cell Biol* 2003, **4**:1.
7. H Kamencic, A Lyon, PG Paterson, BH Juurlink: **Monochlorobimane fluorometric method to measure tissue glutathione**. *Anal Biochem* 2000, **286**:35-7.

A**ppendices**

Items	**Description**
1	Example Excel file showing vitamin E analyses of tissues samples provided by Dr. Dana Anderson.

SECTION 4: Salil Das, DSc, Meharry Medical College

Can Antioxidant Liposomes Protect Lungs from Deleterious Effects of Mustard Gas Exposure

Abstract

The exact mechanism by which mustard gas exposure causes ARDS is not well known. The present study indicates that CEES, a mustard gas analog causes lung injury and significantly decreases expression and activity.of cholinephosphotransferase (CPT), the terminal enzyme in CDP-choline pathway for phosphatidylcholine synthesis. This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating the expression of CPT. We have previously shown that exposure of CEES to guinea pigs causes an increase in the levels of TNF- and NF- B in the lung within an hour. However, NF- B disappeared after 2 hours indicating an intricate interplay of pro- and anti- apoptotic inflammatory cytokines. In the present study, we utilized a state of the art cytokine array technology to identify other cytokines affected by CEES exposure. The array of cytokine induction within an hour of CEES exposure and dynamic changes in cytokine profile by one day post CEES exposure reveals that following an initial damage, the lung tissue tries to recover and prevent further damage through self defense mechanisms mediated through various classes of cytokines.

Introduction and Body

Mustard gas is a poisonous chemical agent that exerts a local action on eyes, skin and respiratory tissue followed by impairment of nervous, cardiac and digestive system in humans and laboratory animals [1-4]. Sulfur mustard disrupts and impairs a variety of cellular activities. Inhalation of mustard gas causes hemorrhagic inflammation to the tracheobronchial tree with severe pulmonary complications including adult respiratory distress syndrome (ARDS) [5]. Most deaths are due to secondary respiratory infections. Besides its use in World war I and World war II, sulfur mustard has been used on Iranian soldiers, on civilians during the Gulf war and on the Iranian-occupied village of Halabja as a vesicant chemical warfare agent resulting in many civilian casualties [6,7]. Mustard agents are also harmful in long-term exposure at low doses. Long term exposure of mustard gas may lead to lung cancer as indicated by the studies on Japanese who worked in poison gas factories [8]. Unfortunately, the molecular mechanisms of carcinogenesis in former poison gas workers remains unclear [9], and the attempts to seek confirmatory and substantial evidence in laboratory animals for links between mustard gas exposure and cancer have not yielded consistent results [10].

Several studies in rats and mice have shown that the mechanism of mustard gas action on lung, skin or other organs includes DNA alkylation; cross linking of DNA [11]; activation of proteases resulting in proteolysis of several vital intracellular enzymes and structural proteins [12]; production of free radicals and free radical-mediated oxidative stress [13,14]; inflammation [15]; and activation of tumor necrosis factor (TNF-α), a part of the inflammatory cytokine cascade [16,17]. It appears that the initiation of free radical-mediated TNF-α cascade is the major pathway in the mustard gas mediated ARDS.

We have established that structurally and functionally, guinea pig lungs are more alike to human lungs in comparison to other animal species [18]. Therefore, we developed the guinea pig model to understand the mechanisms of mustard gas mediated lung injury with particular emphasis on the down stream signal transduction events [19,20]. Our results clearly demonstrate

a complex signal transduction pathway in mustard gas mediated lung injury. After intratracheal injection of CEES to guinea pigs, TNF-α level increased sharply within one hour of exposure. TNF-α level started declining after one hour and returned to basal levels within 24 hours. After the accumulation of TNF-α, both acid and neutral sphingomyelinase activities were stimulated, and both peaked within 4 to 6 hours after CEES exposure. Though both the acid and neutral sphingomyelinase activities were stimulated, the level of acid sphingomyelinase was found to be much higher after CEES exposure. In comparison to lung tissue, lung macrophages contain higher levels of TNF-α and sphingomyelinases and this may be due to fact that lung tissue consists of several types of cells not all of which are responsive to CEES.

As the sphingomyelinase activity increased, there was an accumulation of ceramides. Ceramide levels increased within one hour of CEES exposure. However, there was a slight fall in the ceramide level between 3-6 hours; it increased again at high level even up to 14 days after CEES exposure. It is not known at this time what is the physiological significance of this 14 days elevation of ceramides. The slight drop in the ceramide level between 3 and 6 hours might be due to the activation of NF-kB, which showed a sharp transient activation at 1 to 2 hours after CEES exposure. The activation of NF-kB coincided with the increase of TNF-α in lung tissue.

It is well known that TNF-α is proapoptotic [21] and NF-κB acts as antiapoptotic by opposing the TNF-α induced apoptosis [22-24]. Here, we observed a biphasic effect of CEES on lung. After the initial damage by TNF-α, there was some recovery due to activation of NF-κB within 2 hours. This biphasic pattern was also observed in caspases activation. Significant but small activation of caspase 2, caspase 3, caspase 8 and caspase 9 were observed within 1 hour of CEES exposure. This activation of caspases declined thereafter and reappeared in between 4-6 hours, initiating cell apoptosis in lung as observed by light as well as electron microscopy (paper communicated). This second phase of caspase activation disappeared within 24 hours and we could not observe any further activation of any of the above caspases. This type of biphasic action has been observed in mustard gas induced skin lesions also [25], where an initial phase of injury after 1 hr is followed by a delayed phase which becomes evident after 8 hr of exposure. Our results explain this biphasic action of mustard gas and delineate the events leading to cell death after mustard gas exposure.

Our study thus indicates that CEES exposure causes accumulation of TNF-α which thereby activates sphingomyelinases resulting in the production of ceramides and simultaneous activation of caspases, and finally apoptosis. Ceramides are known to cause apoptosis via activation of caspases (26-28). The present study revealed that there was some initial damage of the lung tissue when exposed to CEES but self-defense mechanism/s of lung tried to recover from the damage and prevent from further damage. The balance between these two opposite effects determines the extent of damage to the tissue. Furthermore, the present investigation enhances our understanding of mustard gas mediated pro-apoptotic signaling pathways and characterizes the events of mustard gas induced lung dysfunction. The results presented here provide a molecular and cellular basis for developing strategies for pharmacological intervention, with potential of clinical application. As the effects of CEES is dose-dependent, it will be beneficial to design the drugs which not only block CEES induced intracellular signal transduction events, but which also directly reduce the contact of CEES on lung surface. We are in progress of developing some aerosols containing a mixture of drugs which will not only prevent the CEES induced signaling events, but also chemically inactivate/modify CEES within lung before it reaches and interacts with the lung cells.

It is known that skin injured by mustard gas exposure can be treated by rapidly decontaminating or detoxifying the contaminated sites and applying ointment to the surface. However, those kinds of treatments are not possible for lung injury and no prophylactic treatment has been available for pulmonary injury by mustards. Due to this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. We have tested several antioxidants and decided to study NAC in details as that was found to be most effective [20]. A single dose of NAC, just before the exposure could not prevent any of the CEES mediated signal transduction events, but pretreatment of animals for 3 days prior to CEES exposure was highly effective in preventing the early signaling steps of CEES-mediated lung injury. Treatment for a longer period (30 days) with NAC provided additional protection. Although the oral administration of NAC could not block all of the CEES mediated signal transduction events, inhibition was sufficient to prevent the ultimate lung damage as observed by histochemical studies. The failure of single dose of NAC just before CEES exposure was not unexpected since systemic levels of NAC were insufficient. This delay offered ample time for CEES to initiate signal transduction and progress of the lung injury.

Protection by NAC from half-mustard gas-induced acute lung injury has also been demonstrated recently in rats by McClintock *et al*. [29]. However, in those studies NAC was administered by liposome encapsulation directly into the lung, as a method of treatment for acute exposure to mustard gas. The mechanism of protection was not elucidated in the studies by McClintock *et al*. [29]. In our study, we have demonstrated that NAC inhibits the production of NF-*k*B. The protection of lung injury thus may be etiologically related to the inhibition of oxidative activation of the transcription factor NF-*k*B, which is usually upregulated by stress signals. In fact, Atkins *et al.* [30] have suggested that NAC protects from sulfur mustard induced apoptotic endothelial cell death by enhancing the synthesis of reduced glutathione, which in turn may scavenge sulfur mustard and also prevent activation of NF-*k*B.

In summary, our study clearly suggests that NAC, a well-known antagonist, can be used as an effective antidote against CEES–induced lung injury. Work is under progress to develop devices to deliver this drug directly into lung even immediate after CEES exposure. Oral administration of NAC, as a prophylactic treatment, for three days or greater has shown significant protection against CEES. Prior to this work there has been no means of prophylaxis against mustards. It would appear that NAC is an excellent candidate prophylactic agent that is inexpensive, non-addicting, safe, and readily obtainable. It is important to note here that there is no other known example of the down regulation of the activity of NF-*k*B that has been shown by the oral administration of an antioxidant. This is important in terms of possible systemic inflammatory pathologic reactions – this point deserves further investigation.

It needs to be emphasized that there were no long-term studies to mimic the clinical or battlefield conditions of mustard gas exposure. Therefore, it is necessary that we continue our efforts to study also long-term effects of mustard gas exposure on lungs.

Key Research Accomplishments

Study 1. **Inhibition of Cholinephosphotransferase Activity in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analog**

Exposure to mustard gas causes inflammatory lung diseases, including acute respiratory distress syndrome (ARDS). A defect in the lung surfactant system has been implicated as a cause of ARDS. A major component of lung surfactant is dipalmitoyl phosphatidylcholine (DPPC) and the major pathway for its synthesis is the cytidine diphosphocholine (CDP choline) pathway. It is

not known whether the ARDS induced by mustard gas is mediated by its direct effects on some of the enzymes in the CDP-choline pathway. In the present study, we investigated whether mustard gas exposure modulates the activity of cholinephosphotransferase (CPT), the terminal enzyme by CDP-choline pathway.

Adult guinea pigs were intratracheally infused with single doses of 2-chloroethyl ethyl sulfide (CEES) (0.5 mg/kg b. wt. in ethanol). Control animals were injected with vehicles only. The animals were sacrificed at different time and the lungs were removed after perfusion with physiological saline.

CPT activity increased steadily up to 4h and then decreased at 6h and stabilized at 7 days in both mitochondria and microsomes (Fig. 1).

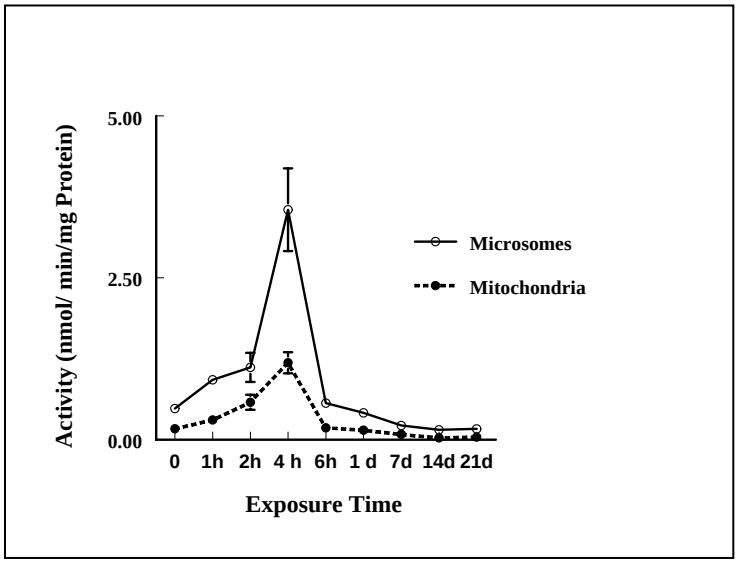
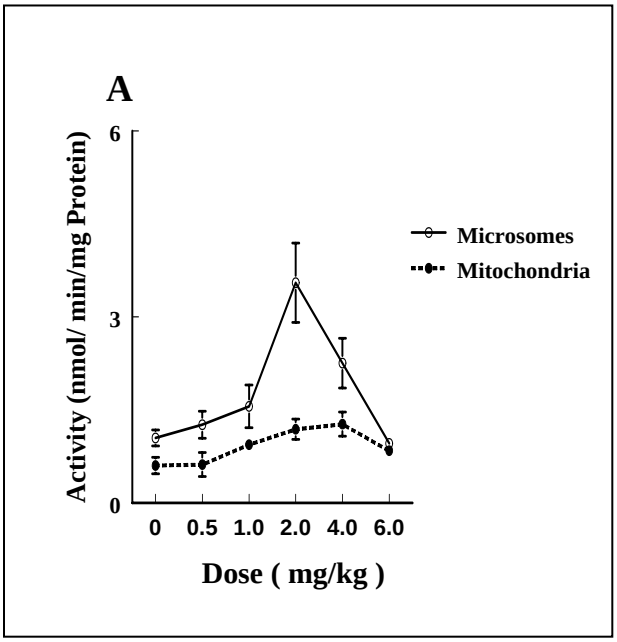


Figure 1. Time dependent effect of 0.5 mg/ kg body weight CEES treatment on mitochondrial and microsomal CPT activity. N=3

To determine the dose-dependent effect of CEES on CPT activity, we varied the dose of CEES (0.5–6.0 mg/kg b. wt.) and sacrificed the animals at 1 h and 4h. CPT activity showed a dose-dependent increase up to 2.0 mg/kg b. wt. of CEES in both mitochondria and microsomes, .and then decreased at 4.0 mg/kg b. wt. (Fig. 2A for 1 h, 2B for 4 h)



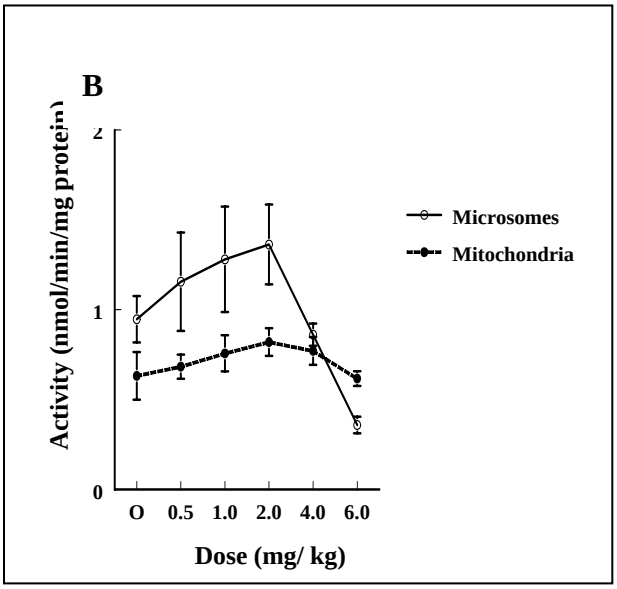


Figure 2. Dose dependent effect of CEES on cholinephosphotransferase activity in mitochondria and microsomes. (A) after 1 h CEES treatment and (B) after 4 h CEES treatment. N=3

For further studies we used a fixed single dose of CEES (2.0 mg/kg b. wt.) and fixed exposure time (7 days). Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue and expressed as the permeability index (Fig. 3).

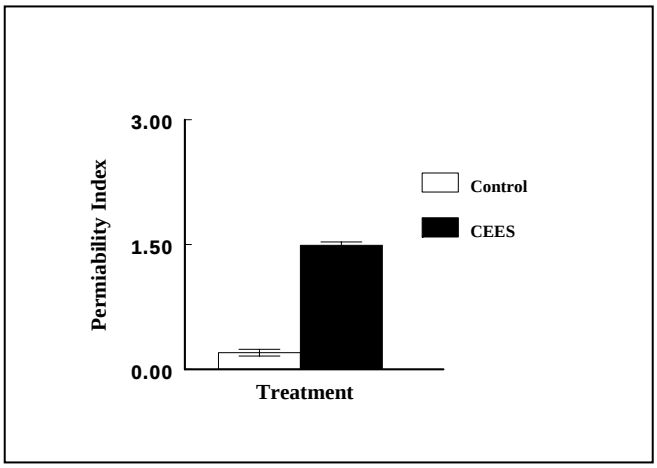


Figure 3. Lung injury measured by leakage of ^{125}I from lung. Animals were exposed to 2 mg/kg body weight CEES for 1 h. $p \leq 0.05$, N=3

CEES exposure (2.0 mg/kg b. wt. for 7 days) caused a significant decrease of both CPT gene expression (~1.7 fold, Fig. 4) and activity (~1.5 fold, Fig. 5) in lung. This decrease in CPT activity was not associated with any mutation of the CPT gene.

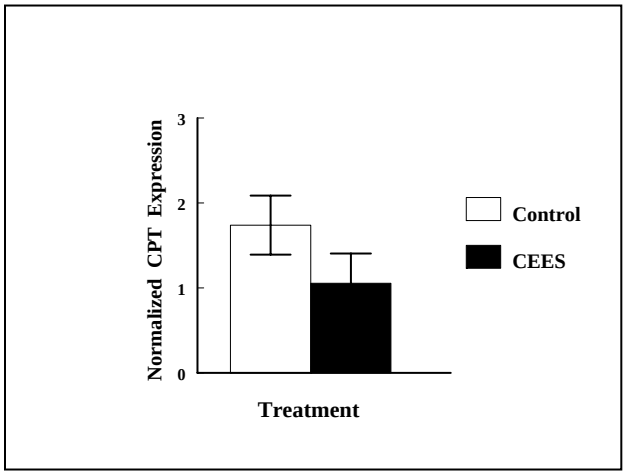


Figure 4. Northern blot analysis for the expression of the CPT gene. Graph showing down-regulation of CPT expression as the result of mustard gas treatment normalized with GAPDH. N =3

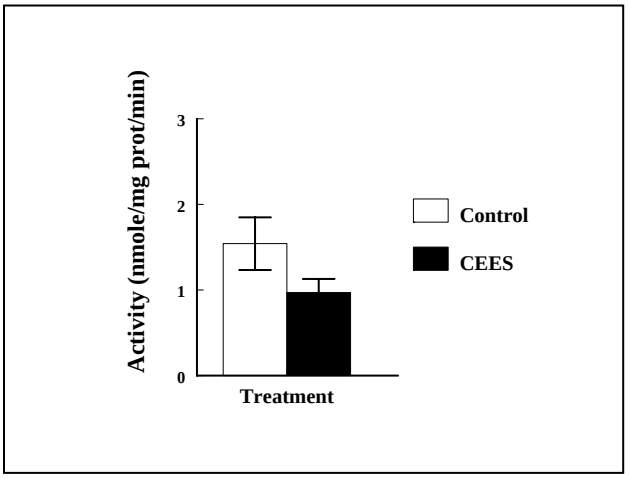


Figure 5. CPT enzyme activity in the lung microsomal fraction of 2 mg/kg body weight CEES (for 7 days) treated lung as compared to the control (only vehicle) showing significant decrease in activity. $p \leq 0.05$, N=5

Previously we reported that CEES infusion increased the production of ceramides, which are known to modulate PC synthesis. To determine whether ceramides affect microsomal CPT activity, the lung microsomal fraction was incubated with different concentrations of C_2-ceramide prior to CPT assay. CPT activity decreased significantly with increasing dose and time (Fig. 6).

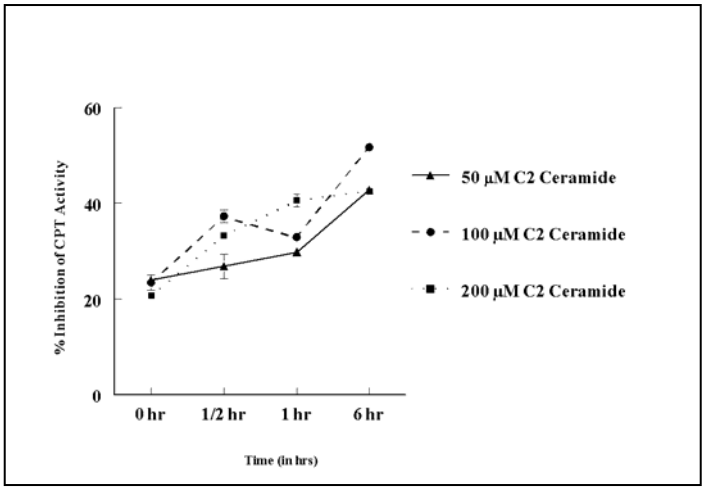


Figure 6. Percent inhibition of the CPT enzyme activity as the result of C_2 ceramide treatment of the lung microsomal fraction. N=3

The present study indicates that CEES causes lung injury and significantly decreases CPT gene expression and activity. This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating CPT enzyme(Fig. 7) .

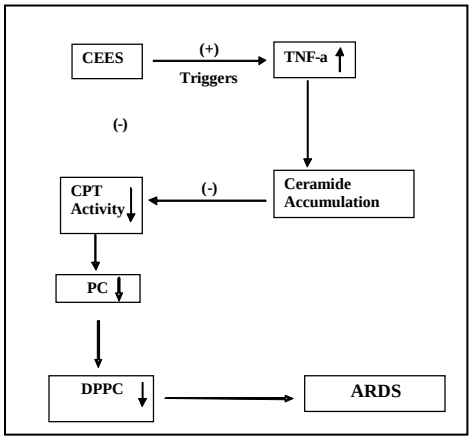


Figure 7. Proposed pathway for induction of ARDS by CEES.

Study 2. Array of Cytokine Induction in Early Lung Injury Response to 2-Chloroethyl Ethyl Sulfide, A Mustard Gas Analog.

We have previously shown that exposure of 2-chloroethyl ethyl sulfide (CEES), a mustard gas to guinea pigs causes an increase in the levels of TNF-α and NF-κB in the lung within an hour. However, NF-κB disappeared after 2 hours indicating an intricate interplay of pro- and anti- apoptotic inflammatory cytokines.

Elucidating the early signaling events initiated in response to mustard gas mediated lung injury, would help us to design early intervention and /or protection against severe lung injury. With this in mind, we utilized the state of the art cytokine array technology to identify cytokines induced in response to mustard gas exposure.

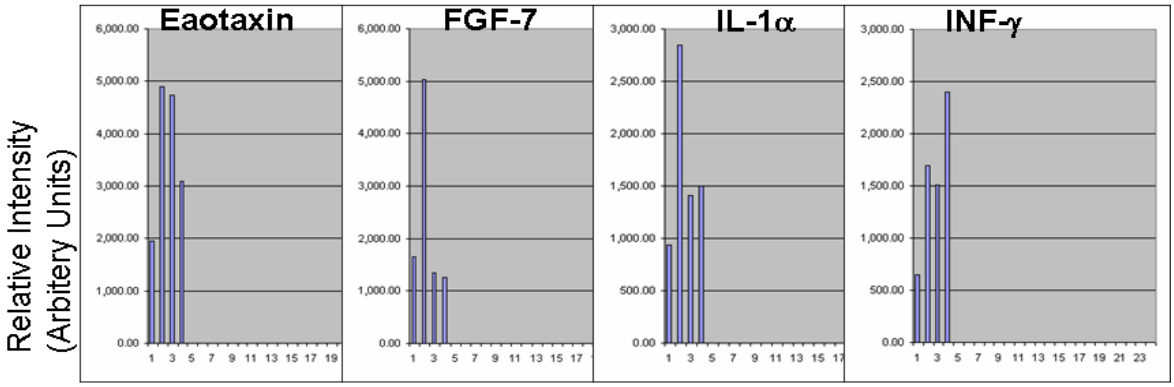


Figure 8. Induction Profile of Selected Cytokines: Columns in graphs indicate (left to right) cytokine induction levels from ; **1**- 1HR Control, **2**- 1HR SMG exposed, **3**- Day1 Control, **4**- Day1-SMG exposed lungs (3 lungs per treatment)

Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs. Among these, 9 cytokines with known or predicted functions in cellular injury and defense signal (IL-1α, EOTAXIN, MIP-1γ), macrophage activation (IFN-γ), inflammatory response (TNF-α), apoptosis (TNF-α), activation of NF-κB (LIGHT), cell proliferation and wound healing (PDGF-BB, FGF-7 and IGFBP-I) were all induced at higher levels with a minimum cut-off point of 2x above the levels of the control lungs (eg; Figure.8). Eotaxin regulated by both TNF-α and IL-1α is also known to be induced in response to radiation. We extended our evaluation to additional 60 cytokines (for a total of 120) at one hour and post mustard gas exposure which identified up-regulation (> 1.5x) of several growthfactors (FGF2) chemoattractant proteins (MCP-3) and cytokines involved in extra-cellular (TSP) remodeling (uPAR and TIMPs). To further understand the dynamics of cytokine induction profile we also evaluated the changes in the levels of these 120 cytokines by one-day post mustard gas exposure.

The array of cytokine induction within an hour of CEES exposure and dynamic changes in cytokine profile by one day post mustard gas exposure reveals that following an initial damage, the lung tissue tries to recover and prevent further damage through self defense mechanisms mediated through various classes of cytokines.

Study 3. Pulmonary Fibrosis in Guinea Pig Induced by 2-Chloroethyl Ethyl Sulfide.

Cross sectional clinical study on veterans with single heavy exposure to sulfur mustard gas (SMG) revealed that inhalation of SMG can lead to the development of series of chronic destructive pulmonary sequelae such as chronic bronchitis, pulmonary fibrosis (PF), and bronchiectasis. To understand the mechanism by which SMG exposure causes PF, we have used 2-chloroethyl ethyl sulfide (CEES) as a SMG analog to induce lung injury in guinea pigs. Our initial electronmicroscopic study revealed that intratracheal exposure of single dose of CEES (0.5 mg/kg b.wt.) developed PF within 7 days (Fig. 9).

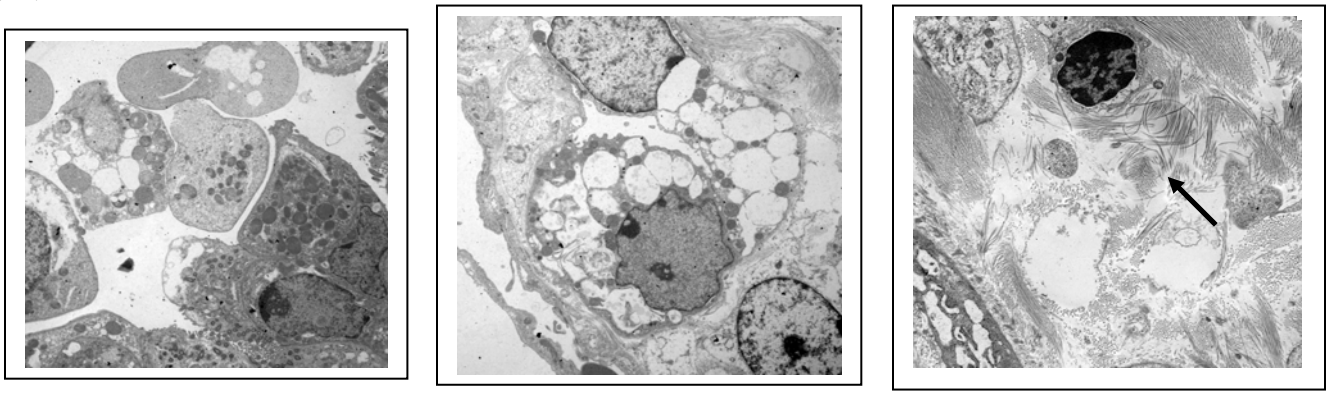


Fig. 9 Electronmicrograph of CEES induced pulmonary fibrosis. Magnification (X 4200)

Macrophage, eosinophils and neutrophils were the predominant cell types in bronchiolar lavage fluid (BALF) as observed in SMG-induced PF patients. The present ultrastructural studies on lung of guinea pigs exposed to CEES indicate evidence of interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen. CEES exposure caused oxidative stress in a time and dose dependent manner in both lung tissue and cells of BALF. Furthermore, there was a significant time dependent increase in MMP-9 (92 Kda gelatinase) activity in cytosol (Fig. 10) and a decrease in vascular endothelial growth factor in BALF (Fig. 11).

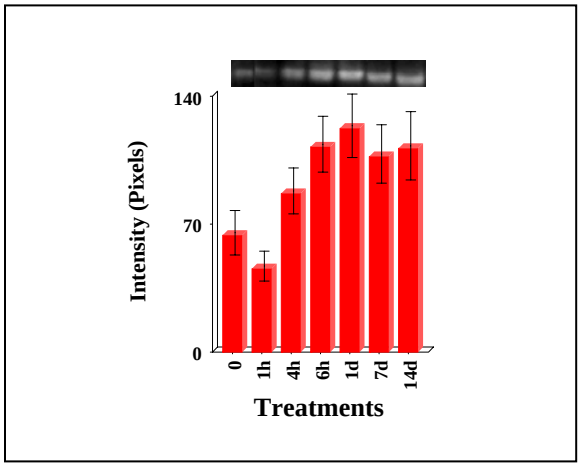


Fig. 10 Time dependent effect of CEES on MMP-9 activity in Cytosol. N=4

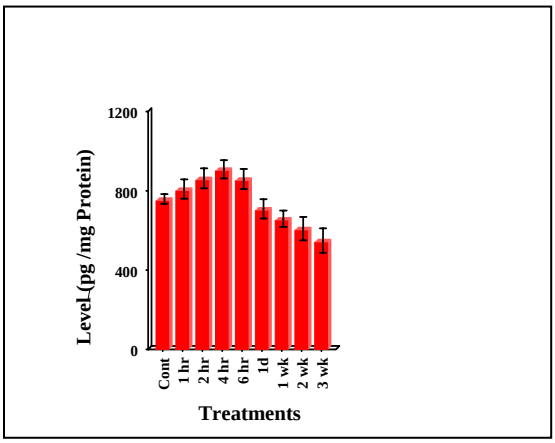


Fig. 11 Time dependent effect of CEES on VEGF level in BALF. N=4

Summary of Key Research Accomplishments

- ❖ CEES exposure significantly decreases gene expression and activity of a key enzyme in phospholipid biosynthesis, Cholinephosphotransferase (CPT). This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating CPT enzyme.
- ❖ We utilized the state of the art cytokine array technology to identify cytokines induced in response to mustard gas exposure. Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs. Among these, 9 cytokines with known or predicted functions in cellular injury and defense signal (IL-1α, EOTAXIN, MIP-1γ), macrophage activation (IFN-γ), inflammatory response (TNF-α), apoptosis (TNF-α), activation of NF-κB (LIGHT), cell proliferation and wound healing (PDGF-BB, FGF-7 and IGFBP-I) were all induced at higher levels with a minimum cut-off point of 2x above the levels of the control lungs.
- ❖ CEES exposure causes interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen without granulation formation. Furthermore, there was a significant time dependent increase in MMP-9 (92 Kda gelatinase) activity and a decrease in vascular endothelial growth factor in BALF.

Reportable Outcomes

Abstracts

1 Mukherjee S and Das SK. Pulmonary Fibrosis in Guinea Pig Induced by 2-Chloroethyl Ethyl Sulfide. FASEB J., Vol. 19, A280, 2005, FASEB Meeting, April 2-6, 2005, San Diego, CA.

2 Rajaratnam VS and Das SK. Array of Cytokine Induction in Early Lung Injury Response to 2-Chloroethyl Ethyl Sulfide, A Mustard Gas Analog. FASEB J., Vol. 19, A852, 2005, FASEB Meeting, April 2-6, 2005, San Diego, CA.

Manuscripts

1. Chatterjee, D., Mukherjee, S. and Das, S.K. Mustard Gas Induced Pulmonary Complications in Guinea Pigs. In: Recent Advances in Molecular Medicine, Allergy and Immunology (Editors: B. Pilo, M.P.Nair, M.S.Patel, C.N.Ramchand), Allied Publisher, Chenai, India, p. 105-116, 2004. (Book Chapter)
2. Chatterjee, D., Mukherjee, S., Smith, M. G. and Das, S. K. Role of Sphingomyelinase in the Environmental Toxin Induced Apoptosis of Pulmonary Cells, in Press, LIPIDS: Sphingomyelin Metabolizing Enzymes" (Volume Editors: D. Haldar and S. K. Das), Research Signpost Publishers; Trivandrum, p. 117-139, (ISBN: 81-7736-229-1), 2004. (Book Chapter).
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Collaborative Arrangements (within Mustard Gas Consortium)

- Direct interactions with Dr. Peter Ward (University of Michigan) to assess by light and transmission electron microscopy structural changes in lungs exposed to CEES.
- Direct interactions with Dr. William Stone (East Tennessee State University) to determine the most protective forms of anti-oxidant liposomes, based on Dr. Stone's *in vitro* studies using cell lines exposed to CEES.
- Direct interactions with Dr. Keith Crawford (Brigham and Women's Hospital, Boston, <A) to assess patterns of gene expression in lungs of guinea pigs after CEES exposure.

Conclusions

- CEES induced ceramide accumulation may play an important role in the development of ARDS by modulating CPT enzyme.
- A state of the art cytokine array technology may be successfully used to identify cytokines induced in response to mustard gas exposure. Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs.
- CEES exposure causes interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen without granulation formation.

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SECTION 5: Keith Crawford, M.D., Ph.D., Center for Blood Research

Utilization of Gene Expression Signatures to Diagnosis Acute Exposure to Genotoxic Agent, 2-Chloroethyl Sulphide (CEES)

Abstract

Our previous work involved the in vitro and in silico screening of transcripts, which are selectively expressed by CEES-exposed circulating blood dendritic cells and monocytes. These transcripts serve as the template for the development of toxicology-base gene expression microarray (ToxArray). Consortium members have access to these arrays for the evaluation of HD induced cellular pathways. Gene expression signatures generated from these studies will serve as the foundation for the development genomic- and protein-based methods of diagnosing exposure to various chemical weapons.

Introduction

Dendritic cells (DC) and monocytes (Mo), principle immune cell residents of the lung, are intimately involved in the maintenance of lung physiology. When exposed to pathogens or chemical agents, these immune cells become activated and release various inflammatory mediators. In addition, these activated immune cells migrate into the circulation and modulate the function of circulating DC/Mo. In extreme cases and depending on the inhaled substance, these lung DC/Mo may induce circulatory collapse (i.e. shock). Because of these unique properties of DC/Mo, our project aim is to identify those genes, which are modulated in human DC/Mo after exposure to CEES. These genes will serve as the foundation for the development of microarrays and the subsequent identification of diagnostic indicators of exposure from HD and other chemical warfare agents. A further goal in conjunction with consortium members is to design rat and mouse microarrays and QPCR reagents, which will be used to assess chemical-specific gene expression in mouse and rat experimental models.

Body

The experiments performed during the reporting period (Aug 03 – Aug 06) have focused on the development of protocols for the enrichment of mRNA and proteins from human DC/Mo. In vitro studies evaluating the effect of CEES, ammonia, hypochlorous acid and azide clearly document agent-specific alterations in protein profiles. Total RNA has been isolated from the treated samples and gene expression analysis of these samples is presently underway. The total RNA from these samples will undergo assessment with full human genome microarrays. Data generated form these samples will be evaluated and affected genes that were not included in our earlier panel will be added to the pool of genes, which will makeup our final ToxArray panel. Following the completion of the above study, the human gene chips will be printed and tested.

Completed project areas

1. Identify CEES-modulated genes and cellular pathways.
2. Development of a tox-gene template for microarray printing .
3. Tissue bank containing tissue samples from HD- or CEES- exposed rats

Continuing studies involve four different areas:

5. Analysis of HD and CEES exposed tissue by gene microarray and protein profiling.
6. Construction of rat and mouse tox-panels.
7. Development of a secure consortium database (DB): Experimental data generated from consortium experiments will be stored in a secure DB. This DB not only allows storage of experimental data, but also will allow viewing and analysis of gene expression data with data mining software.
8. Gene valiadation of unique genes by QPCR.

Key Research Accomplishments (Aug 03 – Aug 06)

- Identification of the cellular pathways, which are responsible for CEES-induced pathogensis. Experimental results suggested that DNA damage induced by DNA adduct formation causes imbalances in the redox state of cells and tissue. This redox imbalance or increase in oxidant levels initiates the caspase pathways.
- Interference of caspase pathways with Pan Caspase inhibitors protects against CEES-induced apoptosis.
- N-Acetylcyteine is more protective against CEES exposure than Pan Caspase inhibitors.
- CEES suppresses proinflammatory cytokine production in by DC/Mo but increases secretion of nitric oxide.
- Development of a panel of tox genes, which allows for the identification of genes, which may serve as biomarkers for exposure to various chemical weapons.

Reportable Outcomes

11. Turnquist, S., Smith, M., Crawford, K. The Effect of Sulfur-mustard on Primary Macrophages. Poster at Bioscience 2004, Hunt Valley, Maryland May 18-20, 2004.
12. Turnquist, S., Fluckiger, R., Smith M., Crawford, K. 2004. CEES-induced Apoptosis in Myeloid Cells. (manuscript in preparation).
13. "APC biosensor: Use of biomarkers for early detection of exposure to chemical or biological agents", presented by K. D. Crawford to Congressional Staffers at Summit Meeting, , Capital Building, Washington DC Oct 18, 2004.
14. APC-biosensor: Use of Dendritic cells and Monocytes for early detection of Exposure to Toxic Substances. 2005 In vitro Biology Meeting, Baltimore, Maryland, June 5-7 2005.

Conclusions

See above.

References

None.

Appendices

None

SECTION 6: Alfred Sciuto, PhD, US Army Medical Research Institute of Chemical Defense

In vivo model assessment of the effects of nebulized sulfur mustard (SM) and CEES in the anesthetized and ventilated rat.

Abstract

This study was designed to establish the following: (1) develop an inhalation model to determine the LCt50 of nebulized mustard and CEES agents in a dose-response manner (2) assess toxicological, biochemical and physiological markers of injury at 0.5, 1, 3, 6, or 24 h post exposure (3) markers of interest include but ae limited to wet/dry lung weight ratio, survival at 24 h and out to 14 days, bronchioloar lavage fluid (BALF) protein and LDH levels. Once these common endpoints are established then antioxidant liposome countermeasures will be administered.

Introduction and Body

1. Model development. The inhalation exposure glovebox became fully operational for animal use in February 1005. Currently we have studied the following nebulized doses of dilute sulfur mustard 0, 250, 500, 750, 1250, or 1750 μg total delivered amount over 10 min via a nebulized stream in the ventilated and anesthetized male rat (260-300 g). XCSM (exempt chemical surety material) HD was diluted from a stock solution into a 75% saline/25% ETOH solution. Nebulized particles were in the respirable range of 1-2 μm. The concentration of HD was verified using the MIN Chemical Agent Monitor (MINICAMS).
2. Determination of markers of exposure. We have not yet achieved a LCt50 for SM. However, we have reached an approximate LCt10 at 1250 μg. Therefore, we will need to complete studies at concentrations that approach 2000 μg (200 μg/10 min exposure). We have determined basic markers of injury at 0, 0.5, 1, 3, 6, and at 24 h postexposure. To this point we have completed only three rats at 1750 μg. There has been relatively little change in wet/dry wt ratios across all time points and doses. BALF protein levels have not shown any marked changes at the lower doses. We did begin to see temporal increases at the 1250 μg level. Protein levels increased 4-fold from 0.5 h to 24 h suggesting a breach in the air/blood barrier is developing over time. Increased BALF LDH levels appear to be dose-dependent at 0.5, 1 and 3 h, but remain inconclusive at 6 and 24 h post exposure. At 1250 μg there is an increased time-dependent trend that is in general agreement with protein levels. Taken together the data thus far suggests that we see some degree of early HD-induced cellular injury 0.5-3 h, but HD-induced distal lung effects as measured by protein levels begin as early as 1 h postexposure and continue to increase out to 24 h. Observationally, we see hemorrhagic lung tissue at necropsy especially in the upper cranial lobes. We have collected unperfused lung, liver, and brain tissues for future analysis. Because we have been diligently investigating the effects of HD, we have not had the time to do similar work with CEES.
3. We have since run into unforeseen difficulties such as senior tech is on continued medical leave, junior tech is pregnant and cannot work with agent, the MINICAMS has been returned to the factory for repairs, and the exposure glove box is down due to cracked glass casing causing pressure fluctuations. It could be a month before we resume studies.

Key Research Accomplishments

See above.

Reportable Outcomes

See above.

Conclusions

See above.

References

None.

SECTION 7: Dana Anderson, US Army Medical Research Institute of Chemical Defense

Comparison Of Antioxidant Liposome Treatment Of Sulfur Mustard or 2-Chloroethyl Ethyl Sulfide Induced Lung Injury

Abstract

Several investigators in the Mustard Consortium have documented protection against 2-chloroethyl-ethyl sulfide (CEES) induced lung injury using n-Acetyl Cysteine (NAC) alone or encapsulated in liposomes. In order to provide additional information for the assessment of liposome encapsulated antioxidants as a treatment for sulfur mustard (SM) induced lung injury we proposed to do a direct comparison between SM and CEES. Once a dose of each compound is established the subsequent evaluation of the antioxidant in liposomes will begin.

Introduction and Body

1. Identify doses of CEES and SM, which yield comparable injury. In the current work, we used the dose of CEES (6 mg/mg) and have determined, via light microscopy, a SM dose to induce a comparable lung injury. To accomplish this anesthetized rats were tracheally intubated and placed them on their backs on an incline. A piece of PE-50 tubing was introduced through the endotracheal tube into the left bronchus, and one hundred microliters of either diluted CEES or SM was slowly infused into the left lung. To prepare the 6 mg/kg dose of CEES, 14.3 ul of CEES was solubilized in 85.7 ul of absolute EtOH and further diluted in 900 ul of Dulbecco's Phosphate Buffered Saline (DPBS). Initially, two doses of SM, 0.7 and 1.4 mg/kg, were evaluated. The stock SM (9.4 mg/ml) was made up in EtOH and then further diluted in EtOH to the final concentration. A new SM stock (8 mg/ml) was made up 50/50 in EtOH and DPBS and further diluted in DPBS to allow a dose of 1.2 mg/kg. At 24 hrs postexposure lungs were removed and formalin fixed for H&E staining and evaluation. All lung lobes (left lung, right cranial, right medial, right caudal, and right accessory) and the trachea were each assessed for extent of damage based on the following parameters: Trachea/chondrocyte necrosis, bronchiolar infiltrates, bronchiolar epithelial necrosis, bronchiolar lymphoid tissue necrosis, alveolar fibrin/edema, alveolar hemorrhage, alveolar cellular infiltrates, alveolar epithelial necrosis, pulmonary congestion, perivascular fibrin/edema, and perivascular cellular infiltrates. Each parameter was given a score of 0 thru 4. (0 being normal.. 1 = minimal; present in 1-10 % of the section. 2=scattered changes; present in 11-25% of the section. 3=moderate; present in 25-45% of the section. 4=severe; present in greater than 45 % of the section.). The exposed lobe and trachea were typically the only sections showing any injury and these data are shown below. A small number of lungs are currently being assessed by electron microscopy. Based on pathologist's light microscopy assessment of the SM exposed lungs, the lung injury was qualitatively indistinguishable from that induce by CEES.

24 hr H&E Pathology comparison between CEES and HD following left lung instillation.

	Lung				Trachea		
	0.7 HD	1.2 HD	1.4 HD	6 CEES	0.7 HD	1.4 HD	6 CEES
Alveolar epithelial necrosis	1.6	1.5	1.7	2.0	*	*	*
Alveolar exudates	2.0	2.3	2.6	2.3	*	*	*
Alveolar hemorrhage	1.4	1.3	1.7	1.7	*	*	*
Alveolar PMN infiltrates	0.3	0.5	0.0	1.0	*	*	*
BALT necrosis	1.2	1.3	2.0	0.7	2.0	2.3	1
Bronchiolar epithelial necrosis	2.1	1.0	2.9	2.3	*	*	*
Bronchiolar exudates	2.1	2.0	2.7	2.2	*	*	*
Bronchiolar PMN infiltrates	0.7	1.0	0.1	0.8	*	*	*
Cartilage necrosis	*		*	*	1.9	1.8	1.6
Perivascular edema	2.0	1.3	1.9	2.3	*	*	*
Tracheal epithelial necrosis	*	*	*	*	1.6	1.3	0.4

A caveat in the interpretation of the table is that the pathology is scored with a categorical score, not on a continuum, so these "average scores" are not true averages. However, they do give a feel for the data and are provided for that purpose only.

2. Evaluation of liposome encapsulated antioxidant. In an effort to prepare for the liposome evaluation studies, a blood sample was taken from animals used in the dose ranging work above, and rats were then perfused with PBS/EDTA to remove remaining blood from tissues. Samples of liver, kidney, spleen and plasma were flash frozen in liquid nitrogen and sent to Drs. Keith Crawford (Brigham and Womens Hospital) and William Stone (East Tennessee State Univ) for analysis. This gave us the opportunity to establish all the methods that will be used. Dr. Stone's group determined the GSH concentration levels in tissues, and also completed alpha and gamma tocopherol determinations on tissue and plasma samples.

It is shown in the graphs that GSH levels detected in the spleen decreased in response to CEES or SM. Interestingly, in both kidney and liver, GSH levels were stable (kidney) or rose (liver) in response to SM but decreased in response to CEES intoxication. There were only 1 or 2 animals in the 1.2 SM GSH group and this data was quite variable. There appeared to be no changes in tocopherol values due to CEES or SM.

Future Studies: Currently awaiting Drs. Stone and Ward selection of the liposome preps that we will be evaluating in this model.

Key Research Accomplishments

See above.

Reportable Outcomes

See above.

Conclusions

See above.

References

None.